Platelets

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Abstract

The ability of platelets to carry out their hemostatic function can be impaired in a wide range of inherited and acquired conditions: trauma, surgery, inflammation, pre-term birth, sepsis, hematological malignancies, solid tumors, chemotherapy, autoimmune disorders, and many others. Evaluation of this impairment is vitally important for research and clinical purposes. This problem is particularly pronounced in pediatric patients, where these conditions occur frequently, while blood volume and the choice of blood collection methods could be limited. Here we describe a simple flow cytometry-based screening method of comprehensive whole blood platelet function testing that was validated for a range of pediatric and adult samples (n = 31) in the hematology hospital setting including but not limited to: classic inherited platelet function disorders (Glanzmann's thrombasthenia; Bernard-Soulier, Wiscott-Aldrich, and Hermasky-Pudlak syndromes, MYH9-dependent thrombocytopenia), healthy and pre-term newborns, acute and chronic immune thrombocytopenia, chronic lympholeukemia, effects of therapy on platelet function, etc. The method output includes levels of forward and side scatter, levels of major adhesion and aggregation glycoproteins Ib and IIb-IIIa, active integrins' level based on PAC-1 binding, major alpha-granule component P-selectin, dense granule function based on mepacrine uptake and release, and procoagulant activity quantified as a percentage of annexin V-positive platelets. This analysis is performed for both resting and dual-agonist-stimulated platelets. Preanalytical and analytical variables are provided and discussed. Parameter distribution within the healthy donor population for adults (n = 72) and children (n = 17) is analyzed.

Keywords

Flow cytometry, immune thrombocytopenia, pediatric platelets, platelet function, thrombocytopathy

History

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Introduction

Bleeding and thrombotic complications resulting from inherited or acquired disorders of platelet-dependent hemostasis are frequently encountered in clinical practice, and are responsible for a major fraction of mortality and morbidity in the modern world. Although specific causes of their complications for each specific case might be related to changes in the platelet number, or platelet functional activity, or the state of the vessel wall, or some other conditions, there is no doubt that the critically important factor needed for physiological hemostatic response is platelet functional activity. Even when the immediate cause of thrombosis or bleeding is due to some other reason than platelet function (e.g. atherosclerosis), its treatment might target platelets, and assessment of platelet functional activity affected by the drugs and by the disease itself could be needed in any case. Finally, determination of the state of platelets is vitally important as an exclusion criterion, e.g. as a part of the diagnostic tree when bleeding turns out to be due to coagulation system defects.

A critical problem in the contemporary field of platelet-dependent hemostasis is reliable and informative evaluation of platelet function [1]. The classical approach in the field is aggregation assays, but they have poor sensitivity and specificity, and are not applicable to low platelet concentrations in thrombocytopenia. This seems to be a critical shortcoming, as the most common and complex conditions are those where platelet dysfunction is combined with decreased platelet count. Platelets seem to be so much in excess of what is needed for hemostasis that it is extremely difficult to have bleeding at high platelet count unless the functional defect

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is something vital like glycoprotein IIb-IIIa deficiency. Flow perfusion chambers have been increasingly useful for research and diagnostics in the field [2], but they are far from being in the acceptably standardized state, and are all after integral assays: they could detect shifts in the hemostatic balance but not the exact cause unless specifically adjusted for the task. Flow cytometry is a very attractive option for detecting molecular changes in platelet function at the single-cell level [3,4], but is not standardized, and has been scarcely used in clinics except for routine glycoprotein level determination, mostly in the field of inherited platelet disorders [5]. There were few attempts to carry out extensive trials with different flow cytometry protocols (like [6]) in order to find optimal conditions and aim to create a thorough system of efficient, clinically validated platelet function testing with inter-laboratory and inter-device agreement.

In a way, one of the reasons behind insufficient understanding of the role of platelet dysfunction in bleeding and thrombosis is exactly the lack of efficient (and standardized) tools to study platelet function disorders. Pediatric hematology is in particular need of such tools. All inherited platelet function disorders are detected in childhood, and acquired disorders are quite frequent in many of pediatric diseases [7]. Here we describe a flow cytometry screening method of comprehensive clinical platelet function evaluation for children [8–11], adults [10,12–14], or platelet concentrate samples [15], which was developed based on our previous non-clinical research experience of platelet functions [16–21]. As an output, it provides the basic platelet "mapping" providing information about all major functions (adhesion, aggregation, granule secretion, and procoagulant activity) for resting and dual-agonist-stimulated platelets.

Method

Materials

Annexin V-Alexa647 and antibodies against P-selectin (CD62P-Alexa647), glycoprotein I (CD42b-PE), integrin αIIβ3 (CD61-PE) and its activation marker (PAC1-FITC) were from Biolegend (San Diego, CA, USA) or Sony Biotechnology (San Jose, CA, USA). Cysteine-containing version of cross-linked collagen-related peptide (CRP) was either kindly provided by Prof. R.W. Farndale (University of Cambridge, Cambridge, UK), or custom-synthesized and purified by VCPBIO (Shenzhen, China) and then cross-linked as described [22]. Its activity was determined for each batch and then controlled monthly as described below. All other reagents were from Sigma-Aldrich (St Louis, MO, USA).

Patients

Patients and healthy volunteers were recruited in either Rogachev Center of Pediatric Hematology, Kulakov Center for Obstetrics, or Botkin City Clinical Hospital. Investigations were performed in accordance with the Declaration of Helsinki under protocols approved by the respective institutions' Ethical Committees, and written informed consent was obtained from all donors and patients, or their parents. All diagnoses were performed according to the national and international guidelines based on clinical, genetic and functional assays independently of flow cytometry. The list of conditions included: childhood Glanzmann's thrombasthenia type I, Bernard-Soulier syndrome, MYH-9 type thrombocytopenia, Wiscott-Aldrich syndrome, Hermasky-Pudlak syndrome, term and pre-term newborns, acute childhood immune thrombocytopenia (ITP), chronic childhood ITP (before treatment and after a month on romiplostim), chronic adult ITP (before treatment and after a month on romiplostim), chronic lympholeukemia (CLL, before treatment and after two weeks on ibrutinib). Patients characteristics are described in Supplementary TableSI.

Protocol description

Venous blood was collected by venipuncture into vacuum plastic tubes with sodium citrate, final concentration 3.8%. The first 2 mL of blood was discarded. Blood was processed as described in Table I. Briefly, 20 μL of blood was diluted 1:20 in HEPES-buffered Tyrode buffer (HBT buffer, 150 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.4 mmol/L Na_2HPO_4, 20 mmol/L HEPES, 5 mmol/L glucose, 0.5% bovine serum albumin, pH 7.4). Platelets were either left intact or loaded with mepacrine (10 μM) for 30 min at 37°C. For

Table I. Flow cytometry protocol.

	Test tubes			
Step 1: Blood dilution				
HBT buffer	400 μL			
Whole blood	20 μL			
	Intact probes		**Probes with mepacrine**	
Step 2: Mepacrine loading				
Diluted blood	320 μL		100 μL	
Mepacrine working solution (200 μM)	-		5 μL	
Incubation	30 min		30 min	
	Test tube 1 (resting 1)	**Test tube 2 (stimulated 1)**	**Test tube 3 (resting 2)**	**Test tube 4 (stimulated 2)**
Step 3: Platelets activation				
Diluted blood	10 μL	10 μL	-	-
Diluted blood with mepacrine	-	-	10 μL	10 μL
HBT buffer with 2.5 mM $CaCl_2$	10 μL	-	10 μL	-
A mixture of CRP + SFLLRN + $CaCl_2$	-	10 μL	-	10 μL
Incubation	10 min	10 min	10 min	10 min
Step 4: Antibody staining				
A mixture of PAC1-FITC, CD42-PE, Annexin V-Alexa647	20 μL	20 μL	-	-
A mixture of CD61-PE, CD62p-Alexa647	-	-	20 μL	20 μL
Incubation	10 min	10 min	10 min	10 min
Step 5: Dilution for flow cytometry				
HBT buffer with 2.5 mM $CaCl_2$	360 μL	360 μL	360 μL	360 μL

platelet activation a mixture of CRP (originally 20 μg/mL, the specific concentration for each lot was adjusted by titration as described below), PAR-1 activating peptide SFLLRN (25 μM) and 5 mM $CaCl_2$ was added to diluted blood in proportion 1:1 (10 + 10 μL) and incubated for 10 min. The appropriate amount of HBT buffer with 2.5 mM $CaCl_2$ was added to the samples without activation. Annexin V-Alexa647 for evaluation of procoagulant platelets and antibodies against P-selectin (CD62P-Alexa647), CD61-PE and PAC1-FITC, CD42b-PE were used. A mixture of antibodies was added to the activated and non-activated samples for 10 min. The samples were then diluted by adding of 180 μL HBT buffer with $CaCl_2$ and analyzed with flow cytometry.

Platelet flow cytometry protocols: brief review and choices

There are several essential variables in the flow cytometry protocols that are briefly reviewed here in order to substantiate choices used in our methodology. Although there are protocols for platelet-rich plasma (PRP) and washed platelets, clinical flow cytometry analysis is usually performed in diluted whole blood [23]. We followed this choice to minimize sample handling time and avoid platelet damage. Blood is usually diluted with buffer such as HEPES-buffered saline to prevent aggregation (and clotting if activation is done in the presence of calcium). We used Tyrode-HEPES buffer as a more physiological medium for platelet activation studies and to keep continuity with our previous studies [17,19,24,25]. Monoclonal antibodies are added to the mixture prior to platelet activation in the lowest saturating concentration determined by titration. Generally, one antibody is used to identify platelets, while another antibody serves as platelet phenotypic marker. In guidance from the SSC of the ISTH it is recommended to use antibodies against major surface glycoproteins (GPIIb/IIIa, GPIIIa, GPIb, GPIb/IX, and GPIIb/IIIa activation epitope) as part of the first-step tests and expanded flow cytometry with additional antibodies (against GPIa/IIa, GPVI and procoagulant activity by annexin V binding) as one of second-step tests in platelet function disorder diagnosis [5,6]; these steps were combined in our protocol aimed at developing a test for all-purpose screening. Various agonists are added to the mixture to induce platelet activation and degranulation. The commonly used ones are adenosine diphosphate, arachidonic acid, collagen, epinephrine, thrombin, thrombin receptor-activating peptide (TRAP) and thromboxane A_2 analogs (e.g., U44619). The concentration and type of agonist depend on the type of assay being performed. We chose dual-stimulation of CRP and TRAP to allow robust and potent stimulation activation employing two major pathways. The activation is stopped by adding large volume of buffer. A fixative can be added to the stopping solution, but it could affect platelet membrane properties and may be wrongly interpreted as part of activation response [23]; we avoided this part. Flow cytometry analysis results can be expressed as percentage of cells that are marker positive or mean fluorescence intensity of whole platelet population; the latter solution was adopted in our approach to obtain values that could be interpreted as number of receptors.

Platelet flow cytometry

Flow cytometry was performed using an ACEA NovoCyte 3000 cytometer (ACEA Biosciences, Inc.) and NovoExpress software version 1.2.4 (ACEA Biosciences, Inc). Flow cytometer day-to-day performance verification, and long-term performance tracking were controlled with NovoCyte Quality control particles (ACEA Biosciences, Inc.) or Sphero Rainbow calibration particles (ACEA Biosciences, Inc.) on a daily basis according to manufacturer's instructions. Automatic sample loading from 96-well microtiter plates was used. Analyzed sample volume was at least 10 μL and slow flow rate (14 μL/min) was used. FSC-H threshold was 2000. Unstained sample and samples stained with single color were used for compensation settings. Samples were analyzed within 15 min after dilution. Platelets were identified according to their typical forward and side scatter properties as well as CD42b PE or CD61 PE binding (Figure 1a). For Glanzmann thrombasthenia (no CD61 binding) or Bernard-Soulier syndrome (no CD42b binding), platelets in the respective run were gated using their forward and side scatter only. One should keep in mind that using CD42b/CD61 fluorescent threshold is preferable in order to better collect small platelet events that could be mistaken for debris in the FSC/SSC gating. For all parameters, the mean values of the gated platelets were determined as the main outcome (e.g. mean SSC, mean CD42b). In order to express them in a device-independent manner, we normalized FSC, SSC, CD42b, CD61, and mepacrine by the mean values for the healthy adult population resting platelets. For normalization of activation markers PAC1 and CD62P, we used the mean values of these parameters as determined for the healthy adult population after dual-agonist platelet stimulation under conditions of our assay. One exception was annexin V binding, where positive platelet subpopulation was clearly distinct (Figure 1a), so its percentage itself served as the outcome. The recommended statistics for sample collection is 5000–10 000 platelet events [26], although it was possible to collect 1000 events in case of severe thrombocytopenia with acceptable reproducibility. Variability of the assay parameters was less than 10% (Supplement Table S2).

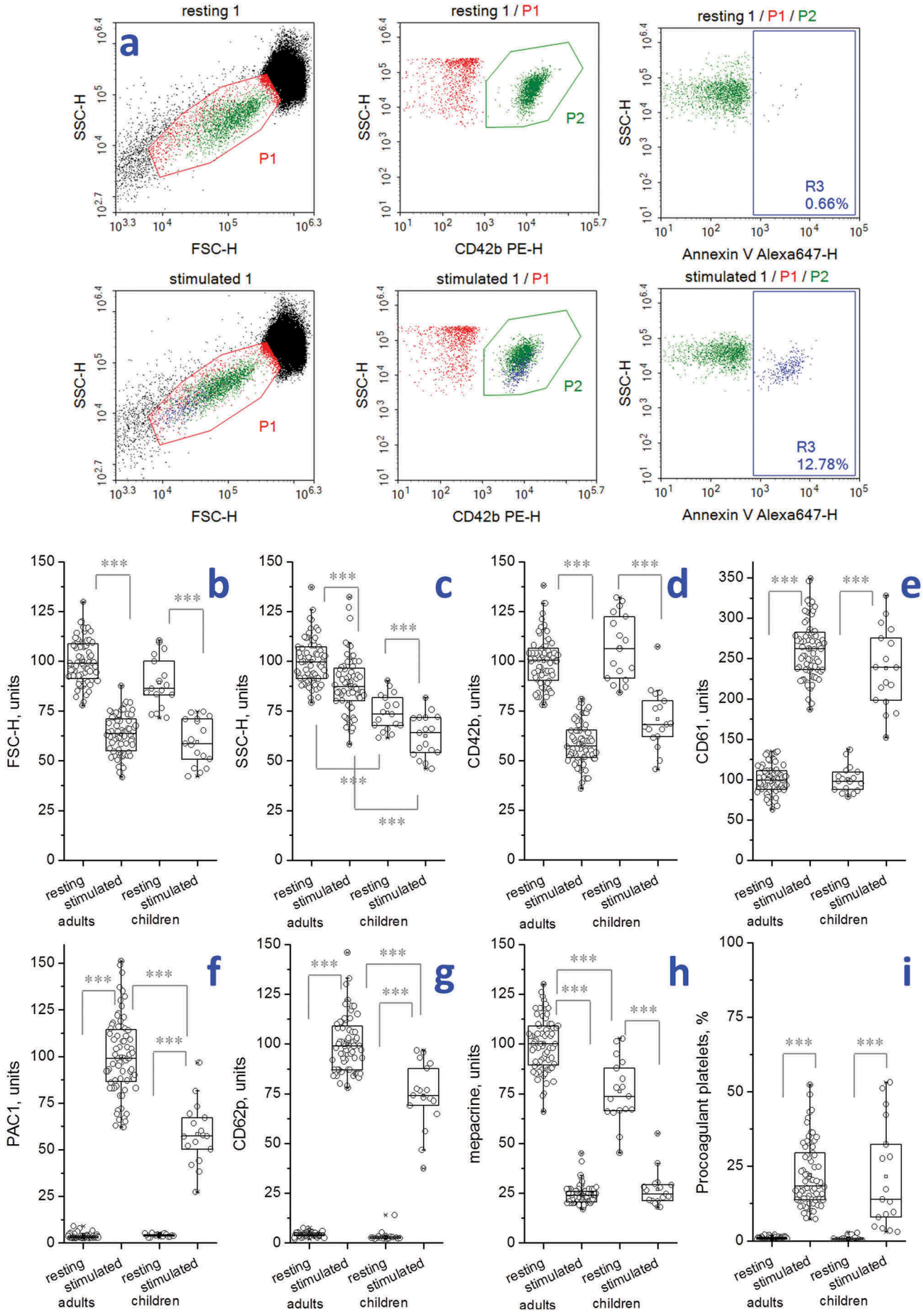
Reagent standardization and transfer of the method between cytometers

Antibodies from different manufacturers (or different lots of the same manufacturer) were calibrated by comparing with the previous one on the same blood sample, and conversion coefficients were determined as described in the online supplement. Transfer between the cytometers was performed by running the same labeled and unlabeled samples on different machines and calculating conversion coefficients; our experience currently includes transfer of the method to BD Accuri, FACS Canto (BD Biosciences), and Navios (Beckman Coulter, Indianapolis, IN, United States). Ability of CRP from different manufacturers or different batches to activate platelets was tested alone and in combination with SFLLRN to adjust working concentration. For monthly control of the reagents and upon reagent change control experiments were performed with blood of four healthy volunteers.

Preanalytical and analytical variables

The results of the assay were not sensitive to the blood/citrate ratio (the results did not differ when citrate concentration was increased three-fold in order to mimic a severe blood collection shortage), and did not differ significantly for freely flowing blood or that collected using citrate tubes of different manufacturers. They remained reproducible within time interval of 15–90 minutes after blood collection if stored at room temperature without disturbance. It should be noted that this is not in line with previous reports on the instability of platelet activation-dependent markers over the course of the first hour [27]; additional research is required to clarify the nature of these differences. Transportation within the city by courier using car or train was acceptable if carried out within an hour, at 20–25°C, with the tubes fixed in a vertical position and without excessive perturbation.

For whole blood platelet count > 20 000/μL, including abnormally high platelet count (patients with thrombocytosis), standard protocol could be used. For lower platelet count, collection time has to be increased. In the case of a very low platelet count (patients with severe thrombocytopenia, PLT < 20 000/μL) PRP should be


a
resting 1
resting 1 / P1
resting 1 / P1 / P2
stimulated 1
stimulated 1 / P1
stimulated 1 / P1 / P2
SSC-H
FSC-H
CD42b PE-H
Annexin V Alexa647-H
P1
P2
R3
0.66%
12.78%
b
c
d
e
f
g
h
i
FSC-H, units
SSC-H, units
CD42b, units
CD61, units
PAC1, units
CD62p, units
mepacrine, units
Procoagulant platelets, %
***
resting
stimulated
adults
children

obtained by centrifugation for 3 min at 100 g (or by blood settling within 20–30 min). To further increase the platelet number in the analyzed sample a lower dilution of whole blood or PRP can be used. The results of the assay were reproducible when whole blood was diluted with buffer 1:15 (instead of 1:21 in standard protocol), but not at 1:10. The assay is not sensitive to the incubation time with the mixture of SFLLRN and CRP (10–20 min).

Method validation with blood of healthy subjects

Blood samples from $n = 72$ healthy subjects claiming not to have used medication for a week prior to analysis were used to investigate the assay properties. In order to evaluate the parameter ranges for pediatric platelets, we collected blood of $n = 17$ children (aged 0–16, median 5 years) who were either healthy or had diagnoses that could be assumed not to affect essentially the platelet parameters (such as leukemia in long-term remission). The assay parameter distribution for healthy adult donors and children is shown in Figure 1b–i and Supplement Figure S1 and Table S2.

Both forward and side scatter in the adults followed Gaussian distribution (at the 95% confidence levels as evaluated with the Shapiro–Wilk and Kolmogorov–Smirnov normality tests) centered at 100 ± 12 units (mean± S.D.). FSC was clearly decreased by 37 ± 7 units upon platelet stimulation, while the effects for SSC were much less clear (11 ± 11 units). Although pediatric platelets had slightly slower FSC (90 ± 12 units), the difference did not reach statistical significance. In contrast, SSC of pediatric platelets was significantly smaller both before (74 ± 9 units versus 100 ± 12 in adults) and after stimulation.

The main adhesion glycoprotein GPIb (CD42b) of the adults followed exactly the same pattern as FSC, with Gaussian distribution of 100 ± 13 units, and almost two-fold decrease on stimulation. The main aggregation receptor integrin αIIbβ3 (CD61) had slightly larger variability (S.D. of 17 units) and was increased 2.5-fold upon stimulation. Distribution of both these receptors in the samples of the children was similar to that of the adults (Figure 1d,e).

The next four parameters described four major activation responses of platelets (Figure 1f-i). PAC1 binding was used as a measure of integrin αIIbβ3 activation. Its binding in the resting platelets was low and did not follow normal distribution (Supplement Figure S1a), so its normal reference range was set as that below 6 units (Supplement Table S2). PAC1 in the stimulated adult platelets was relatively variable among donors at 100 ± 22 units. Likewise, the normal range of P-selectin (CD62P, used as a measure of alpha-granule release) in the intact platelets was set as less than 6.4 units, while its distribution in the activated samples was 100 ± 14 units. For the children's platelets, both PAC1 and CD62 at the resting state were similar to the adult levels, and both were significantly impaired in the activated state (responses were 50–70% of adult ones, $p < 0.001$).

Dense granule function was evaluated based on mepacrine fluorescence both in the resting platelets (100 ± 12 units) and that remaining in the stimulated ones (24 ± 12 units). Under conditions of the assay, therefore, more than three thirds of mepacrine was released. Two derived parameters, mepacrine release determined as their difference (76 ± 12 units) and activation index determined as their ratio (4.4 ± 0.8) were used to evaluate absolute and relative secretion efficiency, respectively. The quantity of mepacrine determined in the resting pediatric platelets was significantly decreased compared with adults (about 75%).

Finally, procoagulant platelets in the resting state followed the PAC1 and CD62P pattern (with normal range set as 0–2 units). However, their distribution in the activated donor samples was not normal (Supplement Figure S1b), and was the widest one among all the parameters. Although some of the children had much lower levels of procoagulant platelets than any of the adults, there was no statistical significance for our samples size (Figure 1i).

Flow cytometry for patients with inherited platelet disorders

Well-characterized inherited platelet function disorders are a classic way to evaluate platelet function assay parameters. Figure 2 shows typical results of our flow cytometry protocol for a set of classic thrombocytopathies. FSC was not affected in GT or HPS reflecting normal platelet size, but was profoundly increased in BSS and even more so in MYH9 in line with the macrothrombocytopenia nature of these disorders, and similarly greatly decreased in microthrombocytopenic WAS. The pattern of FSC distribution for stimulated platelets was similar, and SSC followed it as well. CD42b correlated well with FSC in resting and activated platelets with the exception of BSS where it was naturally absent, making it clearly different from MYH9. The CD61 level also correlated with platelet size in both cases except for type 1 GT where it was absent.

PAC-1 binding was normal in BSS, low in WAS and high in MYH9 (expectable due to their size differences), and absent in type I GT. It was also low in HPS, probably as a result of insufficient feedback activation due to dense granule defects associated with this disorder. Resting and stimulated CD62P was completely in line with platelet size bringing no surprises in these disorders. The same was true for mepacrine uptake and dense granule release except for HPS that had little if any mepacrine uptake. Procoagulant platelets were within the normal range in the resting samples except for BSS and two out of three MYH9 samples; in the stimulated ones, they were curiously low in BSS and HPS.

Flow cytometry for patients with acquired platelet dysfunction

The typical outcomes of the assay for the patients with acquired (or at least non-hereditary) disorders of platelet function are shown in Figure 3. The pattern of assay results for healthy newborns is consistent with previous reports [28]: mostly normal initial parameters, but three out of four responses to stimulation (PAC1, CD62P, and dense granule release) are clearly below the lower boundary of the normal range for adults. The platelet activation responses of the moderately pre-term twins (both with skin bleeding) were only a bit inferior to the term ones. Both acute and chronic childhood ITP (with bleeding) was associated with slight increase of platelet size; however, their increase of FSC, SSC, and CD42b was much less than that in BSS or MYH9. Both of them had platelet pre-activation judging by PAC-1, CD62p, or procoagulant platelets. In the same chronic ITP treated

Figure 1. *Design of the method and reference ranges*. Typical dot plots with data gating (a) used to generate mean values of fluorescence intensity for each parameter, and assay parameter distributions (b–i) for healthy adult donors ($n = 72$) and children ($n = 17$). The time from blood collection to processing was within 30 min. The data points in the box charts are diamond-shapes, square symbols show mean values, horizontal lines are medians, boxes show 25–75 percentiles, error bars show 5–95% intervals, and X symbols show maximal and minimal values. Statistic significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

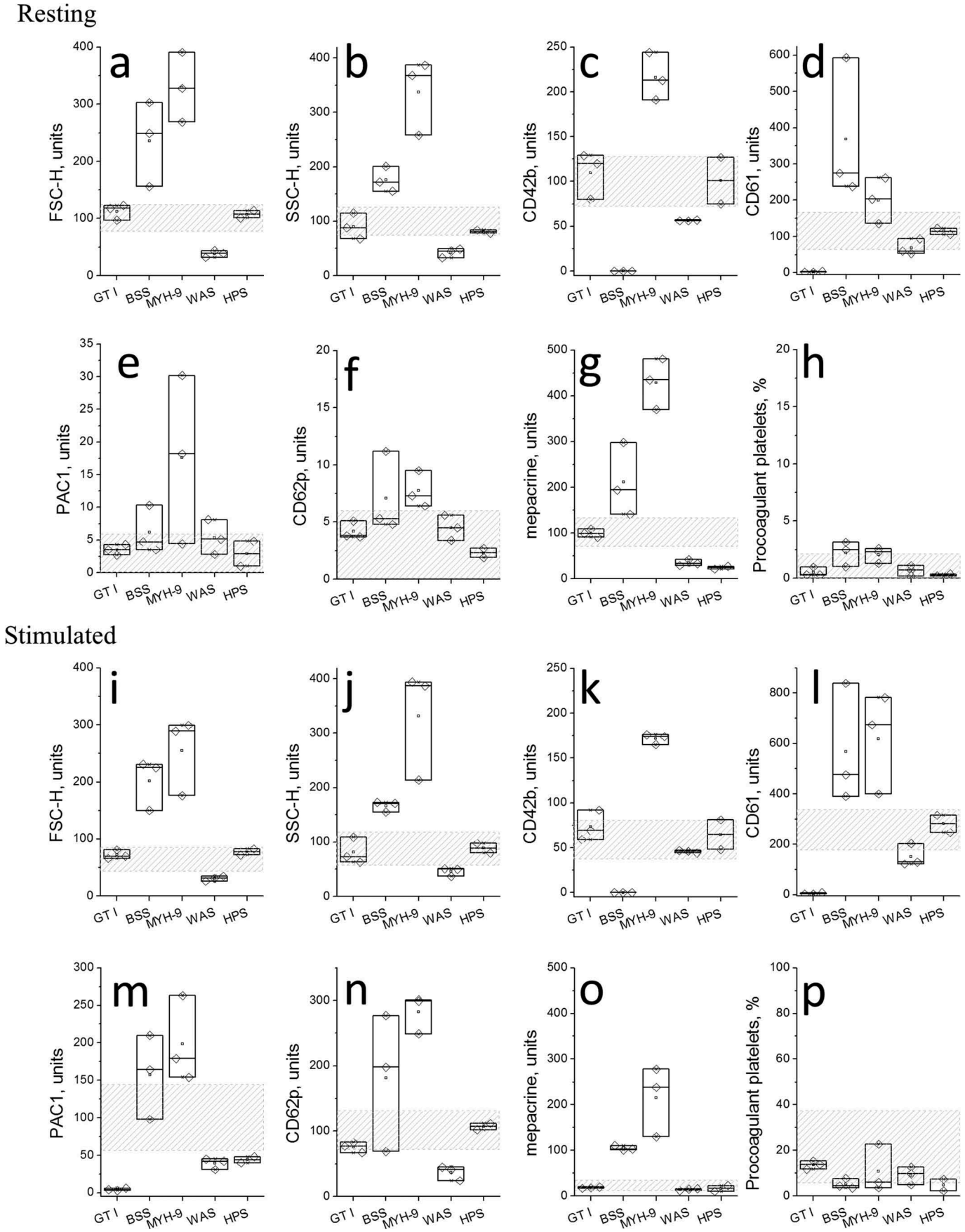


Figure 2. *Inherited disorders of platelet function*. Panels (a–p) show distribution of the indicated parameters for typical patients with classic inherited thrombocytopathies. The patterned regions indicated references ranges. Abbreviations: GT1, Glanzmann's thrombasthenia type 1; BSS, Bernard-Soulier syndrome; MYH9, thrombocytopenia associated with MYH9 mutations; WAS, Wiscott-Aldrich syndrome; HPS, Hermasky-Pudlak syndrome. The time from blood collection to processing was within 30 min.

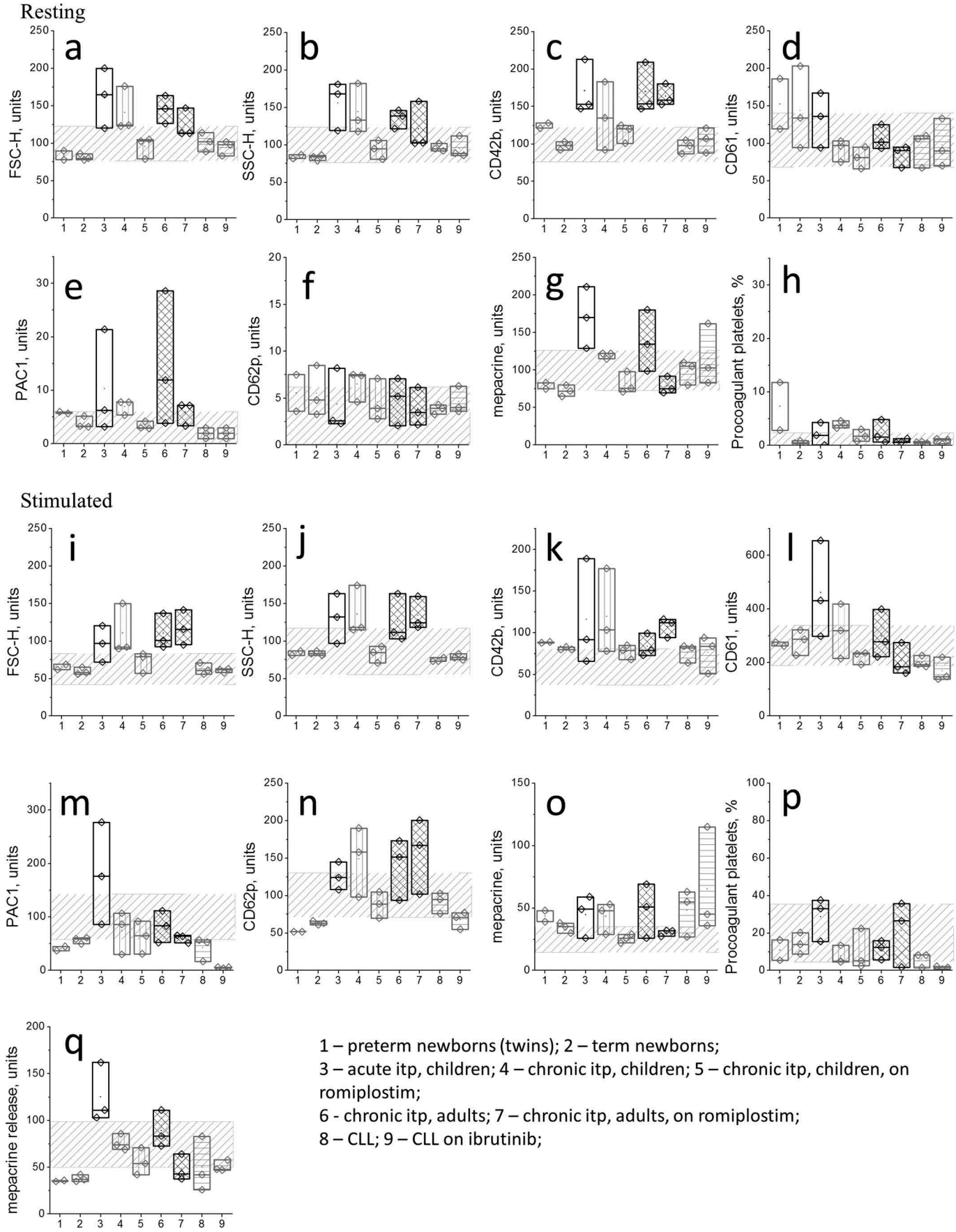


Figure 3. *Acquired disorders of platelet function*. Panels show distribution of the indicated parameters for typical patients with acquired disorders of platelet function. Dotted lines indicated references ranges. The time from blood collection to processing was within 30 min.

with romiplostim, there was not pre-activation, and the size was normal. Adult chronic ITP and its response to romiplostim followed the same pattern. In CLL, platelet response appeared similar to the normal one, but PAC-1 and procoagulant platelets were consistently at the bottom end of the reference range. On ibrutinib, they went further down outside of the normal range.

Discussion

The flow cytometry method of platelet function testing described here was focused on the following goals: comprehensive characterization of all major platelet functions (adhesion and aggregation glycoproteins, integrin activation, release of dense and alpha-granules, procoagulant activity) in the resting and stimulated state, small volume of blood required, minimal preanalytical processing, possibility of using reagents from different manufacturers and almost any two-laser flow cytometer, robust response, and above all sensitivity to function disorders most often encountered in pediatric platelets and being in need of evaluation. Our experience with this method suggests that it is capable of reaching these goals; it is sufficiently robust and reproducible, and it is sensitive to both classic inherited disorders and various acquired conditions.

The data generated by this method with regard to inherited disorders are generally in agreement with previous reports [3,4,6,29]. The levels of glycoproteins absent in the respective disorders (CD61 and PAC1 in GT, CD42b in BSS) were negligibly low, antibody binding and mepacrine loading in all cases correlated with platelet size (giant for BSS and MYH9, small for WAS) except for HPS that did not have dense granules. Although we were not able to reveal any correlations of PAC1 and CD61 within each of the groups, the large-scale differences between healthy donors, BSS, MYH9, and WAS reveal that PAC1/CD61 is maintained at the same ratio suggesting that differences between these disorders are associated with platelet size. One unexpected observation was that giant platelet in BSS had reduced probability of becoming procoagulant under our conditions; this is different from what was reported for BSS before [30].

The results for acquired conditions also agree with some of the published data: some of the main observation reported by other methods before and observed here in our assay include enlargement [31] and pre-activation [32] of platelets in ITP, hypoactivation of neonatal platelets [33], impaired platelet function in CLL [34], inhibitory action of ibrutinib [35]. However, these changes are more difficult to judge, as complexities of these conditions cause controversies in almost of all of these facts (as discussed below for neonatal and pediatric platelets). Additional research is required to clarify these matters. In addition to the data presented here, our preliminary data suggest that for other cases, like pediatric bleeding of unclear origin [11] and quality of platelet concentrates [15], it could also provide clinically relevant outcomes. Impaired PAC1 and PS exposure in HPS and in CLL indicate that the assay is sensitive to the feedback ADP secretion from dense granules.

The indicator markers chosen for the assay are well-characterized in the field [3,6,26] and comprehensive within reasonable limits. While they do not cover the whole range of platelet responses, they cover the major ones. They are subject to the limitations and shortcomings described by others such as potential non-linearity of PAC-1 (compared with fibrinogen binding), non-hemostatic nature of P-selectin (compared with alpha-granular fibrinogen or factor V(a)), or additional preanalytical complications, non-specificity and cytotoxicity of mepacrine. However, these do not seem to be essential under conditions of this assay, and these markers can be easily substituted for alternatives or supplemented with others when needed.

Although the described method was able to identify reproducible platelet function changes for a wide range of pediatric hematology disorders associated with bleeding, the evidence base for its predictive ability with regard to bleeding is very limited. Prospective studies are needed to evaluate its applicability for this purpose. Likewise, its standardization, variability, possibility of reagent modification, characterization of preanalytical requirement are acceptable for a single-laboratory research use, but have not been tested and validated at the multi-center level. Its correlation with other laboratory assays of platelet function is still poorly investigated. In the early screening stage it can be used as a part of diagnostic strategy aimed at the elucidation of the disorder.

This study provides preliminary data on the normal parameter ranges in our assay not only for adults, but also for children aged 1–17 years. The sample size for children is relatively small and does not allow formation of age groups; and additional caution should be exercised because some of the children included were actually patients assumed to have normal platelet function at the time of analysis. Despite these limitations, these results allow deeper interpretation of the results with patients and could be a useful supplement for assay usage. While some of the pediatric parameter ranges are similar to the adult ones, their SSC and mepacrine uptake in the resting state as well as PAC1 and CD62 expression upon activation are decreased. Even greater hyporeactivity was observed in the few neonatal samples of this study. This hyporeactivity of neonates and older children agrees well with some of the previous reports [36–38] but not with others reporting hyper reactivity or no difference related to adults [33,39,40]. Although detailed analysis of these differences is beyond the scope of the present study, it could be speculated that they are due to the use of high agonist concentrations in this study and in [36], while previous reports of hyper-reactivity employed low agonist concentrations [40]. It is worth noting that use of a super-maximal stimulation condition is not capable of demonstrating hyper-reactivity (activation cannot be increased above 100%) and can only measure hypo-responsiveness.

Our results with adult hematology patients seem to be in line with the children. This method however has not been extensively tested outside of the hematology field, so its applicability to many of the problems of adult hemostasis (e.g. arterial thrombosis and most importantly monitoring of antithrombotic drugs) has not been tested. It can be expected, however, that strong stimulation used in this method together with potent blood dilution might make it difficult to sense subtle changes caused by modern antiplatelet drugs (that would be additionally diluted unless they act covalently). Some of these problems might be resolved by modifying assay conditions (less potent activation, different calcium), but it is quite possible that the price to be paid could be loss of robustness.

Conclusions

Here we describe a flow cytometry-based method of comprehensive platelet function testing that validated for a range of pediatric and adult samples in the oncohematology hospital setting. The method output includes levels of forward and side scatter, CD42b, CD61, CD62P, dense granule level and release, annexin V and PAC-1 binding for both resting and dual-agonist-stimulated platelets. Preanalytical and analytical variables are provided and discussed, and parameter distribution within the healthy donor population is analyzed.

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Declaration of interest

The authors report no declarations of interest.

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Supplementary material

Supplementary data can be accessed here

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